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Review article

Mesenchymal stem cells: characteristics and clinical applications

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Abstract: Mesenchymal stem cells (MSCs) are bone marrow populating cells, different from hematopoietic stem cells, which possess an extensive proliferative potential and ability to differentiate into various cell types, including: osteocytes, adipocytes, chondrocytes, myocytes, cardiomyocytes and neurons. MSCs play a key role in the maintenance of bone marrow homeostasis and regulate the maturation of both hematopoietic and non-hematopoietic cells. The cells are characterized by the expression of numerous surface antigens, but none of them appears to be exclusively expressed on MSCs. Apart from bone marrow, MSCs are located in other tissues, like: adipose tissue, peripheral blood, cord blood, liver and fetal tissues. MSCs have been shown to be powerful tools in gene therapies, and can be effectively transduced with viral vectors containing a therapeutic gene, as well as with cDNA for specific proteins, expression of which is desired in a patient. Due to such characteristics, the number of clinical trials based on the use of MSCs increase. These cells have been successfully employed in graft versus host disease (GvHD) treatment, heart regeneration after infarct, cartilage and bone repair, skin wounds healing, neuronal regeneration and many others. Of special importance is their use in the treatment of osteogenesis imperfecta (OI), which appeared to be the only reasonable therapeutic strategy. MSCs seem to represent a future powerful tool in regenerative medicine, therefore they are particularly important in medical research.

Key words: Mesenchymal stem cells (MSCs) - Osteogenesis imperfecta - Gene therapy

Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic cells, which reside in the bone marrow together with better known and characterized class of stem cells - hematopoietic stem cells. They were first described by Fridenstein *et al.* in 1976, as the clonal, plastic adherent cells, being a source of the osteoblastic, adipogenic and chondrogenic cell lines [38]. The interest in MSCs rapidly grows with expanding knowledge about their exceptional characteristics and usefulness in the clinic. This review describes the latest data about MSC biology and behavior *in vitro*, as well as *in vivo*. It presents also molecular features of MSCs and their broad use in various clinical settings.

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Sources of MSCs

The main source of MSCs is the bone marrow. These cells constitute, however, only a small percentage of the total number of bone marrow populating cells. Pittenger *et al.* showed that only 0.01% to 0.001% of mononuclear cells isolated on density gradient (ficoll/percoll) give rise to plastic adherent fibroblast-like colonies [96]. The number of MSCs isolated from this tissue may vary in terms of the yield and the quality, even when the cells are obtained from the same donor [95].

Apart from the bone marrow, MSCs are also located in other tissues of the human body. There is an increasing number of reports describing their presence in adipose tissue [43], umbilical cord blood, chorionic villi of the placenta [54], amniotic fluid [122], peripheral blood [133], fetal liver [11], lung [57], and even in exfoliated deciduous teeth [85].

The amount of MSCs decreases with age [36] and infirmity [56]. The greatest number of MSCs is found in neonates, than it is reduced during the lifespan to

about one-half at the age of 80 [36]. As for circulating fetal MSCs, the highest number is detected in the first trimester and declines during the second trimester to about 0.0001% and further to 0.00003% of nucleated cells in cord blood [11].

Surface markers on MSCs

MSCs constitute a heterogeneous population of cells, in terms of their morphology, physiology and expression of surface antigens. Up to now, no single specific marker has been identified. MCSs express a large number of adhesion molecules, extracellular matrix proteins, cytokines and growth factor receptors, associated with their function and cell interactions within the bone marrow stroma [28]. They also express a wide variety of antigens characteristic for other cell types, as confirmed by advanced molecular techniques, including serial analysis of gene expression [111] and DNA microarray [61]. The population of MSCs isolated from bone marrow express: CD44, CD105 (SH2; endoglin), CD106 (vascular cell adhesion molecule; VCAM-1), CD166, CD29, CD73 (SH3 and SH4), CD90 (Thy-1), CD117, STRO-1 and Sca-1 [5, 7, 21, 26, 44, 160]. Interestingly, the observations made by Bonyadi *et al.* [8] present late-onset osteoporosis in mice lacking Sca-1. Parallelly, MSCs do not possess markers typical for hematopoietic and endothelial cell lineages: CD11b, CD14, CD31, CD33, CD34, CD133 and CD45 [96]. The absence of CD14, CD34 and CD45 antigens on their surface create the basis to distinguish them from the hematopoietic precursors [5]. In Figure 1 we present the phenotype characteristic of the 2nd passage BM-MSCs. This data from our laboratory confirm the standard description of these cells.

MSCs are also known to express a set of receptors associated with matrix- and cell-to-cell adhesive interactions, like integrins $\alpha_V\beta3$ and $\alpha_V\beta5$, ICAM-1, ICAM-2, LFA-3 and L-selectin [21, 96, 7].

There have been studies to find an accurate combination of a limited number of antigens in order to isolate pure population of MSCs from a tissue. From the data available up to now, several options have been proposed in this context. One of them suggests that the co-expression of CD105 and CD73 could be sufficient [96]. Another one implies that the expression of CD166 and CD105 makes it possible to separate the earliest precursors of MSCs from more mature cells [2]. In turn, examination of the CFU-F obtained from bone marrow stroma demonstrated that the MSCs fraction may by identified by several markers, including STRO-1, Thy-1, CD49a, CD10, Muc18/CD146, as well as with the antibodies to receptors for PDGF (platelet derived growth factor) and EGF (epithelial growth factor) [5, 8, 26, 44, 96, 98].

Although MSCs have been described by a subset of surface antigens, little is known about fresh or nonexpanded MSCs, mostly because of their very low frequency in adult bone marrow [7]. The findings by Boiret *et al.* [7] showed that the most discriminative markers for MSCs examined after short time of adherence (1-3 days) were: CD73 and CD49a, as all the CFU-F-colonies (100%) were CD73- and most (95.2%) were CD49a- positive. Interestingly, these data did not confirm that CD105 and CDw90 could be selective markers for MSCs, as only 45.4% and 49% of the CFU-F were positive for these molecules, respectively [7]. Furthermore, the authors checked the surface protein expression on freshly isolated bone marrow MSCs, showing, as found previously, that CD73 and CD49a were the most extensively expressed antigens in CFU-F-enriched subset. These results stand in opposition with the popular description of MSC phenotype, which postulated the STRO-1 antigen to be exclusively expressed by primitive mesenchymal precursors [26, 44].

However, the presence of some antigens may change *in vitro*, due to specific culture conditions and the duration prior to individual passages [22]. Interestingly, some antigens may be found on freshly isolated MSCs, but their expression disappears in culture. Such a phenomenon was observed in case of CD34 antigen. This molecule was expressed by MSCs obtained from mouse fetal lungs, but could not be found in *in vitro* cultures of MSCs [36]. This would suggest that the expression of that molecule vanishes during the maturation process. Similar results were obtained in case of chemokine receptor expression on human MSCs [49]. The second passage BMSCs expressed: CCR1, CCR7, CCR9, CXCR4, CXCR5 and CXCR6. At the 12-16th passage, there was no expression of any of those molecules, which was also confirmed by a disability of the cells to migrate towards specific chemokine attractants. Moreover, the loss of these receptors' expression was accompanied by a decrease in the expression of adhesion molecules - ICAM-1, ICAM-2, VCAM-1 and CD157. Moreover, the alteration in BM-MSCs phenotype was associated with increasing cell cycle arrest and induction of the apoptotic pathway [49].

The change in antigen expression has been also described for MSCs undergoing differentiation process. As an example, the CD166 antigen (activated leukocyte cell adhesion molecule) has been presented on undifferentiated MSCs but was absent from the cells that underwent osteogenic differentiation [10]. Furthermore, the cell clones derived from different tissues may slightly differ in terms of cell surface molecules. A survey investigating the antigen profile on MSCs isolated from adipose tissue revealed that in majority these cells are very much alike as bone marrow-derived MSCs [64]. However, in a small number

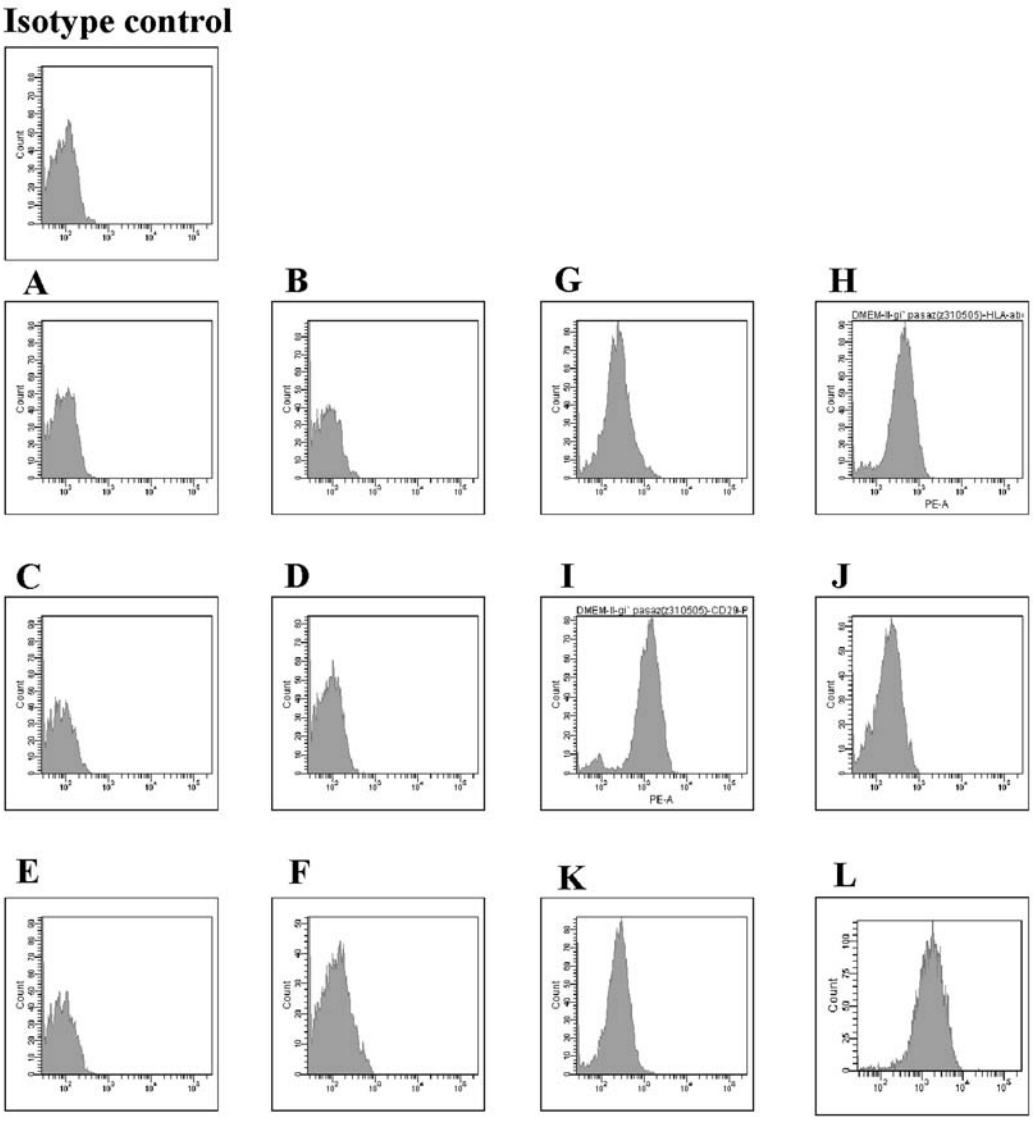

Fig. 1. Phenotype of bone marrow derived MSCs after two passages. Cells were cultured in DMEM with 10% FBS. **A** - CD14, **B** - CD33, **C** - CD133, **D** - CD45, **E** - CD34, **F** - HLA-DR, **G** - CD105, **H** - HLA-ABC, **I** - CD29, **J** - CD44, **K** - CD166, **L** - CD73. Flow cytometry.

of surface proteins, the two populations differ. The adipose tissue MSCs were shown to possess additionally CD49d [64], CD62e and CD31 [43].

Basic biology and functions of MSCs

Human MSCs are known to constitute a heterogeneous population of cells and their properties and functionality depend on the environmental characteristics. MSCs can be expanded in culture were they give rise to fibroblastic colonies (CFU-F). The CFU-F units are well documented to possess an extended proliferative potential *in vitro* [22]. Studies in rodents with ^{3}[H]-thymidine labeling demonstrated that CFU-F are essentially in a noncycling state *in vivo* [133]. The number of colonies obtained from bone marrow aspirates differs among species, as well as throughout the culture conditions used in each individual experiment. Colony formation by MSCs derived from adult human BM is feeder cell independent, while the rodent cells require a source of irradiated feeder cells to achieve maximal plating efficiency [9, 97].

The cultures of MSCs are, however, not completely explored. Former studies claimed that MSCs isolated from bone marrow comprise a single phenotypic population forming symmetric, spindle-shaped colonies (homology up to 98%) [96]. More recent studies, however, indicate that single-cell derived colonies are morphologically heterogeneous, containing at least two different cell types: small spindle-shaped cells and large cuboidal or flattened cells [9, 55]. In terms of proliferative potential, the cells have been also described as small rapidly-renewing, and large slowly-renewing [102]. Contrastingly, the work

performed by Colter *et al.* [19] describes the population of small and agranular cells (RS-1) within stationary culture of MSCs with a low capacity to generate colonies and non-reactive to the cell cycle-specific antigen Ki-67. That cell subpopulation was shown, however, to be responsible for the capacity of the whole population of MSCs to expand in culture. Furthermore, it was speculated that RS cells may cycle under stimulation by factors secreted by the more mature MSCs. These cells were, thus, proposed to represent an *ex vivo* subset of recycling uncommitted mesenchymal stem cells [19].

Nevertheless, the latest findings show that MSC colonies contain as much as three types of cells. The third fraction was described to be composed of very small rapidly self-renewing cells [20], which are reported as the earliest progenitors and possess the greatest potential for multilineage differentiation. The examination of these cells revealed that they were about 7 μm in diameter and had a high nucleus-to-cytoplasm ratio. They could be also distinguished from more mature cells by the presence of specific surface epitopes and expressed proteins, like vascular endothelial growth factor receptor-2, tyrosine kinase receptor, transferrin receptor and annexin II (lipocortin 2). Some of the rapidly renewing cells contained also other markers, like c-kit (CD117), multidrug resistance epitope and epithelial membrane antigen. Interestingly, these cells were negative for STRO-1, an antigen originally considered as a marker for MSCs [26].

MSCs play a significant role in bone marrow microenvironment. The major function of these cells is to create a tissue framework, which assures a mechanical support for hematopoietic cell system. They secrete a number of extracellular matrix proteins, including fibronectin, laminin, collagen and proteoglycans [28]. Moreover, MSCs produce hematopoietic and non-hematopoietic growth factors, chemokines and cytokines, thereby participating in the regulation of hemopoiesis. MSCs secrete: IL-1a, IL-1b, IL-6, IL-7, IL-8, IL-11, IL-14, IL-15, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-SCF), leukemia inhibitory factor, stem cell factor (SCF), fetal liver tyrosine kinase-3, thrombopoietin and hepatocyte growth factor (HGF) [7, 20, 22, 44, 64]. Some of these proteins are produced by quiescent cells, whereas the others after stimulation. The involvement of MSCs in hematopoiesis is additionally consolidated by their presence in fetal liver and bone marrow just prior to the onset of definitive hemopoiesis at those sites [11, 80]. An animal model study confirmed that human MSCs marked with GFP and transplanted into the tibia of NOD/SCID mice, integrated into the functional components of hematopoietic microenvironment and actively participated in the hematopoietic cell development [86]. During 4 to 10 weeks after transplantation, GFP-MSCs differentiated into pericytes, myofibroblasts, stromal cells, osteocytes and endothelial cells. This led to the increase in the number of functionally and phenotypically primitive human hematopoietic cells in murine bone marrow microenvironment. The engrafted cells supported human hematopoiesis via secreted factors and by physical interactions with primitive hematopoietic cells [86]. Other studies showed that cotransplantation of human MSCs and HSCs resulted in increased chimerism or/and accelerated hematopoietic recovery in animal models and in humans [36, 67, 71]. Moreover, MSCs are known to produce a variety of cytokines that are involved in homing (stromal derived factor-1 - SDF-1) or proliferation and differentiation of hematopoietic cells (GM-CSF, SCF, IL-6) [48]. It has been proposed that several chemokine axes are involved in maintaining bone marrow homeostasis, and that some chemokines, which MSCs possess the receptors for, like CCR9 and CXCR4 may operate in an autocrine manner, similarly as it is in case of HSCs [49].

Among other well known biological activities of MSCs, it is worth to emphasize their immunomodulatory functions. These cells are able to inhibit responses of alloreactive T lymphocytes. They express neither MHC class II molecules nor costimulatory receptors (CD80, CD86) on their surface, therefore they do not exhibit antigen-presenting cell activities [3, 36]. The addition of interferon-γ (IFN-γ) to the cultures of MSCs enhances the expression of MHC class I and triggers the expression of MHC class II, but not of the costimulatory molecules. [36]. It has been well established that MSCs from various species can exert profound immunosupression by inhibiting T-cell responses to polyclonal stimuli [29] and to their cognate peptide [69]. The inhibition did not seem to be antigen specific and targeted both primary and secondary T-cell responses [69]. The inhibitory effect was shown to be directed mostly at the level of cell proliferation. T cells stimulated in the presence of MSCs were arrested in the G1 phase as a result of cyclin D downregulation [41]. The suppression, however, was not apoptotic and could be reversed. In the absence of MSCs and with appropriate stimuli, T cells continue to proliferate [29]. The precise mechanism by which MSCs modulate immunological response is still to be clarified, but overall data suggest that soluble factors as well as cell contact mediated mechanisms are involved. Blocking experiments with the use of neutralizing monoclonal antibodies against transforming growth factor-β (TGF-β) and HGF suggest that these factors are at least in part responsible for the inhibitory effects caused by MSCs [29]. Moreover, MSCs can affect other cells participating in immune response like B cells [41] and dendritic cells [63].

Circulation and niches of MSCs

Little is known about the nature and localization of undifferentiated multipotent MSCs. These cells may be found in various tissues in special places called 'stem cell niches', which serve as stem cells reservoirs. They remain quiescent and possess the capacity for self-renewal after an injury, disease or aging [96]. The stem cell niche hypothesis for the bone marrow cells was developed by Schofield, who suggested that certain microenvironmental conditions of the marrow stroma could maintain the stem cells in a primitive, quiescent state [112]. The investigation of anatomical distribution of MSCs within bone marrow revealed that the cells are located in a close association with endosteum [44]. Such places, therefore, could be regarded as potential niches for MSCs. The findings are, however, based on the STRO-1^+ stromal cell population, and the identification of MSCs expressing other specific markers, may change this picture.

The question how MSCs maintain their undifferentiated state within the niche is not completely resolved. However, there are some findings indicating that MSC decision to differentiate or to stay quiescent is regulated by Wnt family members, which support undifferentiated state of MSCs, as well as their inhibitors, like: Dickkopf-1 (Dkk1), Frizzled b-1 (Frzb-1) or sFRP1 [106]. Wnt signaling is known to prevent differentiation process by inducing high levels of oct-3/4, rex-1 and the homeodomain transcription factor Nanog [106]. Apart from Wnt- and Dkk1-mediated signaling, also Notch, Hedgehog and BMP-pathways play a role in proliferation and differentiation of stem cells. Therefore, it can be speculated, that at least some of these factors are also important for MSCs growth in their niche.

After particular stimuli, a stem cell may leave its niche and circulate in blood [35]. The cell must be afterwards attracted to another site, where under specific microenvironmental circumstances is able to enter its differentiation program [127]. The study on MSC homing indicates that the expression of chemokine receptors, as quoted previously, help them in trafficking to various tissues, including bone marrow [76]. Among them, a pivotal role is played by CXCR4, the receptor for SDF-1, which, *inter alia*, is produced by stromal cells. Many findings confirm the extensive multi-organ homing ability of MSCs. In murine model, circulating mesenchymal progenitors, detected in bloodstream, were able to migrate and colonize various tissues [39]. Similar results were obtained in humans [101]. Moreover, these cells were present in the blood of breast cancer patients after growth factor-induced mobilization of hematopoietic stem cells [35]. These data suggest that adequate stimuli may mobilize and release quiescent MSCs residing in a tissue. Additionally, a subset of quiescent cells (5-10%) was identified in cultures of mesenchymal cells isolated from cord blood, suggesting that uncommitted mesenchymal progenitors circulate during gestation, and travel from fetal sites into other tissues early during development [80]. As another example, MSCs were described to locally migrate to injured sites, to support the regeneration process. Such cases were documented in cartilage repair [14, 40], muscle [23] and heart [110] regeneration, migration throughout forebrain and cerebellum [68] and differentiation into osteoblasts in regenerating bone [50, 51]. The homing capacity of MSCs may decrease after extensive culturing *in vitro*. A study based on syngeneic mouse model revealed that primary bone marrow-derived MSCs were able to home efficiently to the bone marrow and spleen, whereas culture-expanded MSCs had lost this capacity after 24-48 hours in culture [36]. It might be speculated, therefore, that *in vitro* propagation of bone marrow-derived MSCs dramatically decreases their homing to bone marrow and spleen.

Growth and expansion of MSCs

Various protocols have been developed to grow and expand MSCs. Cells which initially adhere to the tissue culture plastic, display fibroblastic appearance and develop into symmetrical colonies between 5 and 7 days after plating. Human MSCs proliferate most rapidly and maximally retain their multipotential ability when cultured at relatively low densities [107]. These cells may be seeded at the range from 1×10^4 to 0.4×10^6 cells/cm^2 [37, 82]. The initial culture concentration affects not only growth of MSCs but also their morphology [121]. When the cells are grown at a low density, they mostly display a spindle-like shape, but when they reach confluence and start to grow in several layers, the cells become flat with torn ends.

In vitro growth of MSCs is characterized by the occurrence of three phases, similarly to other progenitor cells: (i) an opening lag phase, which lasts for 3-4 days, followed by (ii) a rapid expansion (log phase) and closes with (iii) a stationary phase [9, 20]. The last stage does not rely on cell contact inhibition and replating the cells triggers their growth for approximately five more passages [20]. Prockop *et al.* [42] suggests that the shift between different stages is regulated mainly by the expression of Dickkopf-1 (Dkk-1) and Wnt5a genes, which play opposite roles. The greatest expression of Dkk-1 appears during the log phase and shortens the former stage by inhibition of Wnt5a expression, whereas Wnt5a protein level becomes maximal during the stationary phase.

Under optimal conditions, MSCs can be maintained in culture for 20-30 population doublings and still retain their capacity for differentiation [37]. More

recent studies show that these cells are able to grow and divide for even more than 50 population doublings [98]. This indicates a great proliferative potential of these cells. Examination of the cell cycle profile of MSCs revealed that about 10% of these cells occurs in phases S, G2 and M of the cell cycle, while the vast majority of the cells remain in the G0/G1 phase [21]. In genomic assays, MSCs maintain a normal karyotype and telomerase activity, even at passage 12 [96]. However, extensive subcultivation of MSCs impairs their functionality and the cells display evident signs of senescence and/or apoptosis [21].

Proliferation of MSCs is influenced by a number of cytokines and growth factors. The list of hormones and other molecules involved in the regulation of CFU-F proliferation *in vitro* is growing. PDGF and fibroblast growth factor-2 (FGF-2) have been shown to be potent mitogens for CFU-F [9], and EGF exerts the same effect on STRO-1 enriched population of MSCs [9]. Opposite results can be obtained after addition of interferon-alpha and interleukin 4 to the culture [9, 61]. Both cytokines inhibit colony formation stimulated by the combination of EGF and PDGF in a dose-dependent manner. Additionally, it was demonstrated that binding of heparin-binding epidermal growth factor (HB-EGF) to its receptor HER-1 on MSCs, consolidates proliferation and prevents differentiation of these cells induced by conditioning [70]. Thus, it can be speculated that the HB-EGF/HER-1 axis is important for MSC renewal and differentiation. The proliferative activity of MSCs was shown to be directly proportional to their differentiation potential [97].

Differentiation potential of MSCs

It is still not clear if there is one multipotent MSC that gives rise to each cell of mesenchymal origin, or a mixture of progenitor cells committed to different cell lineages. *In vitro* and animal implant studies did not solve this problem up to date, showing different, often opposite results [9, 25]. In earlier studies it was believed that MSCs could differentiate only into tissues of mesodermal origin. Recently, according to large-scale studies on MSC biology, this dogma has been changed. Successful differentiation has been achieved in a variety of cell lineages, including osteoblasts, chondrocytes, adipocytes (Fig. 2), fibroblasts, myoblasts and cardiomyocytes, hepatocytes, tenocytes, cenocytes, and even neurons [33, 62, 80, 96, 128]. However, some scientists hypothesize that generating cells of origin different than mesodermal, is due to specific reprogramming process of gene expression in MSCs [105] or occurs as a result of particular soluble factor activity [117]. According to the former hypothesis, it was believed that MSCs undergo a process called 'stem cells plasticity', changing their lineage commitment. One of such theories, termed 'stochastic repression/induction model, claims that differentiation potential observed for various sets of MSCs arises from a series of gene silencing events occurring during development [27]. This results in the appearance of diverse MSC populations capable of expressing different cell-commitment genes. However, the data from other investigators rebut a statement about MSC plasticity [100]. In addition, there are assumptions that the observed change in MSC phenotype results from spontaneous fusion of those cells with other lineage cells [97, 116]. Other authors describe the presence of cell population similar to MSCs called multipotent adult progenitor cell (MAPC) in adult bone marrow [62], which can be obtained together with MSCs during isolation. Culturing MAPCs in specific conditioning media leads to their differentiation into cells derived typically from the three germ layers: ectoderm, mesoderm and endoderm, as also confirmed in the animal models. It is not clear what is the relationship between MSCs and MAPCs. It can be speculated, that MAPCs are either MSC progenitors or just compose an artificial cell population arisen in *in vitro* culture [22]. Besides that, MSCs were shown to express genes specific for both: ectodermal and mesodermal cells, and even for terminally differentiated cells, like neurons and osteoblasts [33]. This data was confirmed using RT-PCR and DNA microarray techniques.

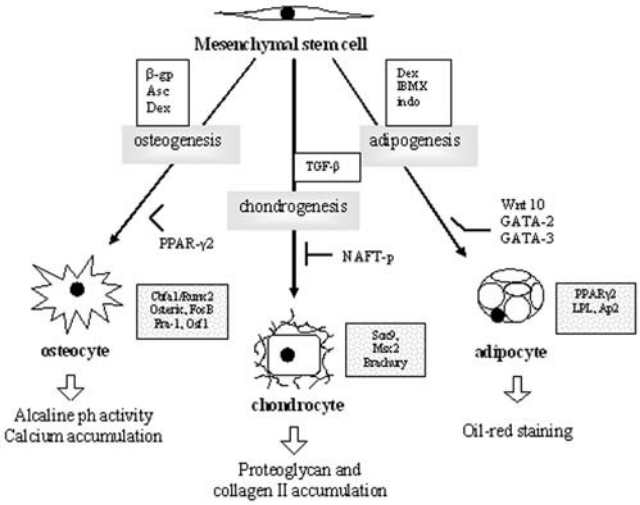

Fig. 2. The scheme of MSC differentiation into the three mesenchymal lineages: osteocytes, chondrocytes and adipocytes. The upper boxes contain inducing factors for each of these pathways, and the lower ones - the major transcription factors (shadowed). Ways to identify differentiated cells are pointed by empty arrows. Abbreviations: β-gp - β-glycerophosphate; Asc - ascorbic acid; Dex - dexamethasone; TGF-β - transforming growth factor-β; IBMX - isobutylmethylxanthine; indo - indomethacin; PPARγ2 - peroxisome proliferation-activated receptor γ2; NAFT-p - nuclear factor of activated T cell; LPL - lipoprotein lipase; aP2 - fatty acid-binding protein.

However, not all the adherent CFU-F colonies obtained from the bone marrow aspirates display pluripotent capacity for differentiation. Pittenger *et al.* [96] reported that approximately one-third of them might be successfully directed to the osteogenic, chondrogenic and adipogenic lineages. *In vitro* differentiation into particular cell lineage demands treating the cells with a proper mixture of specific differentiating factors. It must be mentioned that basal nutrients, cell density, spatial organization, mechanical forces, growth factors and cytokines, all play a role in MSC differentiation. To achieve efficient outcome of the process, each factor should be optimized. Interestingly, the same factor may launch differentiation to diverse cell lineages in cell cultures derived from various species. For example, dexametasone is an established factor that triggers the differentiation towards osteoblastic cell lineage in human MSCs [59], whereas in mouse-derived MSCs it causes adipocyte formation [24]. Contrariwise, recombinant human bone morphogenetic protein 2 (rhBMP-2) at low doses induces mouse MSCs into osteogenic lineage [25], but to obtain the same effect on human MSCs, very high doses of this factor are required [60]. Apart from that, the downstream molecular events are very much alike in various species, which was demonstrated for osteogenesis. Both, human and mouse MSCs involve the transcription factor Cbfa1/Runx2 in this process [32]. It is also known that MSCs synthesize and secrete specific cytokines and growth factors, and the induction into each differentiation pathway involves modulation of their production, as well as regulation of particular signal-transduction pathway proteins [58]. Moreover, the cell density has been also shown to be a critical parameter for differentiation [19, 102]. When MSCs are seeded at low density, they proliferate and secrete Dkk1, which favors the undifferentiating phenotype of the cells. On the contrary, when the cells reach confluence, Wnt-5a expression abrogates the effect of Dkk1 [42].

It has also been reported that the differentiation potential may differ in the relation to the source of MSCs. This statement, however, have as many pros as cons. According to one study, MSCs derived from adipose tissue possess the impaired ability to differentiate into both osteoblasts and chondrocytes [55]. Other scientists, on the contrary, demonstrated that MSCs isolated from fat display the same characteristics as MSCs from bone marrow and might be alternatively used for clinical trials [66].

In order to obtain ***osteoblastic*** cell line, the confluent monolayer of MSCs should be incubated with a mixture containing β-glycerophosphate, ascorbic acid and dexamethasone, throughout the period of 2-3 weeks [27]. Participation of bone morphogenetic proteins (BMPs) in bone formation process has been also postulated, although different BMPs play different roles [15, 60, 71, 89]. Other important factors involved in osteogenic regulation are: TGF, insulin-like growth factor (IGF), brain-derived growth factor (BDGF), FGF-2, leptin and parathyroid hormone related peptide (PTHrP) [79]. These proteins regulate secretion of matrix proteins and the expression of signals necessary for bone remodeling through osteoclast activation. Among the transcription factors committed to osteogenesis, pivotal roles are attributed to Cbfa1/Runx2, Osterix, ΔFosB, Fra-1, Aj18 and Osf1. Apart from them, Msx2, Dlx5 and TWIST were shown to take part in this process [132]. As it was documented, Cbfa1/Runx2 is necessary for osteoblast formation, but only Dlx5 allows distinguishing the mineralized osteoblasts. Progression of osteogenesis might be measured through alkaline phosphatase activity and calcium accumulation (Fig. 3B) [96]. Human MSCs were shown to possess a great potential to differentiate into osteoblasts, which was maintained for up to 40 doublings in culture, even after cryopreservation [9].

Chondrogenesis in turn is classically carried out in micro-mass cultures of MSCs after addition of TGF-β. Among TGF-β family members, the most important role in chondrogenesis play BMPs and cartilage-derived morphogenetic proteins (CDMPs) [27]. Apart from BMP signaling, cooperation between BMPs and members of Hedgehog family (Hh) has been reported [129]. A regulatory role in this process has been attributed to the proteins from Wnt family. Among them, Wnt-4 and Wnt-14 were shown to display high expression at sites of future joint development [34], whereas Wnt-7a was shown to inhibit chondrogenesis [53]. Additionally, as recent data indicate, the signaling triggered by the FGF receptor 3 is sufficient to induce chondrogenic differentiation [48]. TGF-β and related cytokines exhibit the ability to induce signal transduction pathways specific for chondrogenesis, mostly via activation of mitogen-acivated protein (MAP) kinases such as: ERK-1, p38, PKC and Jun [108], whereas FGF receptor acts through Smad protein signaling [48]. The activation leads to induction of specific transcription factors expression. The most important roles play Sox9, Msx2 [109], and Brachury [48]. They were shown to activate the expression of chondrocyte-specific genes, like aggrecan and collagen II. Participation in this process has been also shown for Hox, Pax and Forkhead. Chondrogenic formation, except from morphological changes, may be verified by histological testing for the presence of proteoglycan in the extracellular matrix and collagen type II chains, which are typical of articular cartilage [96]. Inhibitory effect on chondrogenesis may be achieved through nuclear factor of activated T cell - NAFT-p activity [99].

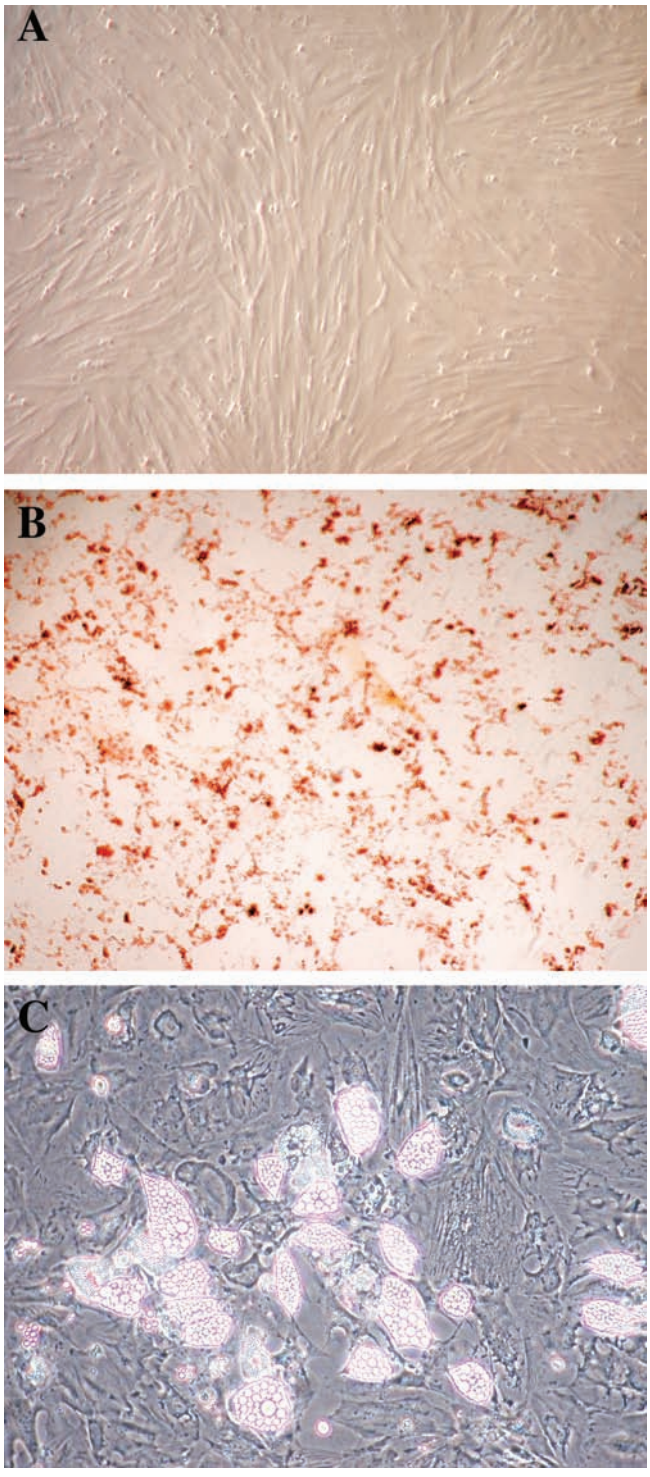

Fig. 3. Bone marrow derived MSCs. Cells were cultured in MesenCult Basal Medium for three passages and then differentiation was started. A - Cultured MSCs; B - MSCs after 20 days of osteogenic differentiation (alkaline phosphatase staining); C - MSCs after 20 days of adipogenic differentiation (note adipocytes containing lipid droplets).

In vitro ***adipogenesis*** (Fig. 3C) can be induced by treating MSCs with a hormonal cocktail containing dexamethasone, isobutyl methyl xanthine (IBMX) and indomethacin [25, 27]. The differentiation might be confirmed using oil-red staining technique and controlling the expression of specific proteins, such as peroxisome proliferation-activated receptor γ2 (PPARγ2), lipoprotein lipase (LPL), and the fatty acid-binding protein aP2 [96]. Inhibition of adipogenesis can by accomplished by the induction of Wnt10b [103], GATA-2 and GATA-3 [120].

An interesting role in MSCs differentiation toward osteoblastic versus adipogenic cell lineage is played by BMP proteins. The BMP-2 as well as bFGF have been shown to synergistically enhance *in vivo* bone formation by MSCs [60, 89]. Selective blocking of the BMP receptor type 1B (BMPR-1R) resulted in the differentiation into adipocytes, which would likewise suggest that the expression of this receptor is required for osteocyte formation. Conversely, overexpression of BMPR-1A blocked adipogenic differentiation and prompted osteoblastic generation [13]. The findings indicate that changes in the BMP receptor levels are intrinsic factors for the commitment into adipogenic or osteoblastic cell line. Additionally, adipocyte transcription factor -PPARγ2 was demonstrated to repress osteogenesis [75].

Apart from factors inducing differentiation into the three cell lines described above, the molecules promoting other cell lineage formation, like myocardium and even neurons, have been identified, but they are not completely defined so far [33, 45, 80]. Moreover, MSCs cultured in each of differentiation conditions produce autocrine and paracrine factors that might be essential for lineage progression [96].

Clinical application of MSCs

The specific characteristics of MSCs, including their extensive proliferative potential and the ability to differentiate into various cell types, like bone, fat and cartilage, make them an attractive tool in regenerative medicine. This is especially evident in such fields as cellular biology and gene therapy, resulting in considerable increase in the number of clinical trials based on the use of MSCs. Apparently, these cells might be simply isolated from various tissues and expanded in culture in large numbers that gives the opportunity to create a tissue-engineered constructs containing these cells and re-introduce them into a patient [104, 124, 131]. Full healing is a complex process and demands integration of the regenerated tissue with the surrounding host tissues and differentiation through the natural signaling pathways. As it was documented, MSCs possess the capacity to engraft into various tissues and organs when infused systematically, and this engraftment has been shown to be stable in the long term [28, 31]. Even more, MSCs infused to the peripheral circulation have the ability to migrate to a specific site of

injury. This phenomenon has been reported in animal models of bone fracture, cerebral ischemia and myocardial infarction [110, 125]. In one study, the authors managed to localize MSCs transplanted to neonatal mice, using the whole body imaging technique [88]. On the 7th day post injection, the cells presented a wide distribution throughout the body of the recipient mice. 18 days later, the majority of infused cells were found in lungs and liver, and a very small population was present in other tissues. Finally, 35 days post infusion, a significant number of the cells was located in bones, indicating that these cells may participate in bone formation [88]. Interesting results were delivered by Prockop *et al.* [76], who examined the MSC engraftment efficiency in various tissues in immunodeficient mice, using a sensitive RT-PCR method. The engraftment appeared to be at a very low level, and varied in different tissues. Interestingly, the survey revealed the presence of a subpopulation of small size MSCs - rapidly-self renewing MCSs (RS-MSCs), which engrafted preferentially in comparison to a larger, slowly renewing MSCs (SR-MSCs). The two subpopulations varied not only in terms of differentiation potential but also in the surface epitopes. The more effective engraftment of RS-MSCs might be partially explained by their expression of CXCR4 and CXCR1, which are known to be involved in the trafficking of MSCs [76].

MSCs have been also proposed to be an excellent potential tool for gene therapies. They can be subjected to various genetic modifications, such as transduction with viral vectors carrying a therapeutic gene or cDNA for special proteins, serving as molecular transmitters. In a mouse model, the genetically modified MSCs implanted in an ectopic site and subsequently transplanted to a secondary donor, showed about 74% stable gene transfer efficiency [31]. They could be therefore useful in delivering particular genes into organs or a tissue of special need. Furthermore, there have been clinical studies in humans with MSCs transfected with viral vectors containing the gene for coagulation factor VII or IX, in case of haemophilia treatment [18]. These cells are also metabolically active and may serve as a suitable source secreting therapeutic proteins, such as defective enzymes [123]. When successful, this approach could bring outstanding results in tissue and body repair.

One of the fields for MSC use in regenerative medicine is the treatment of bone defects. First approach to bone repair relied on biodegradable scaffolds impregnated with recombinant BMPs, and was designed to induce bone formation through the recruitment of local MSCs [71]. This project was successfully accomplished in an animal model (Lewis rats), showing that MSCs attracted to BMP-2 are able to regenerate the injured bone. Such approach was made also by Petite *et al.* [94], who managed to heal large segmental bone defect in sheep. The results were, however, not completely satisfying because the amount and the quality of regenerated bone remained disappointing. As another example, MSCs were activated through the intramuscular injection of adenovirus-mediated hBMP-2 gene transfer in nude mice, which resulted in local MSC proliferation and differentiation [78]. Furthermore, a portion of implanted cells were competent themselves to respond to the factors in an autocrine or paracrine way. The bone healing using MSCs might be improved with the use of other specific cytokines, like IGF, PDGF and FGF [15].

With reference to numerous clinical trials using MSCs, a special attention ought to be paid toward osteogenesis imperfecta (OI) treatment. This is a genetic disorder resulting from mutations in collagen I gene, causing many abnormalities especially in bone structure [52]. There have been over 150 mutations responsible for the OI outcome identified, affecting COL1A1 and COL1A2 genes [84]. As collagen is the major protein of the extracellular matrix of the bone, the patients with OI suffer from frequent and numerous fractures, progressive deformities of limbs and spine, retarded bone growth and short stature [52]. Therefore, a treatment strategy for OI is mainly aimed at improving bone strength through ameliorating the structural integrity of collagen [52]. Among therapies applied to OI, only cell and gene regimens brought positive effect and seem to be the only reasonable tools.

The cell therapy approach targeted to osteoblast formation from MSCs was first investigated on murine models. MSCs isolated from transgenic mice were transplanted into irradiated recipient mice [93]. The location of these cells was inspected 1-5 months after cell infusion. According to the results, 1.5%-12% of the cells were found in various tissues, including bones [93]. Other studies were performed using immunodeficient SCID mouse model, confirming the homing capacity of hMSCs to the bone marrow and the ability to differentiate into osteoblasts *in vivo* [92].

The first steps in therapeutic approach using MSC transplantation in OI patients were done by Horwitz *et al.* in 1999 [51]. Allogenic unmanipulated bone marrow from HLA-identical or single-antigen-mismatched siblings was transplanted to three children with OI. The therapeutic outcome was successful (1.5%-2% of engraftment), showing donor-derived MSCs located in the bone marrow of the recipient. Bone marrow MSCs were able to give rise to properly functioning osteoblasts, resulting in the increase in bone mineral content, as well as the improvement in growth velocity and the reduction of bone fracture frequencies [51]. Encouraged by the results, the authors performed next trials [52]. Bone marrow was obtained

from allogenic, HLA-compatible, sibling donors and was given twice to each patient. Among the five children enrolled in this study, three appeared chimeric and showed donor osteoblast engraftment. As a result, those children gained significant increase in total body length with a median of 7.5 cm, measured 6 months after transplantation, in comparison to 1.25 cm for control patients. Moreover, the bone mineral content improved by 45% to 77% of the baseline values. The number of fractures, visualised by radiography, declined from an average of 10 during 6 months before treatment, to 2. Unfortunately, the follow-up study demonstrated that the growth ratio either decreased or remained unchanged. In contrast, bone mineralization continued to increase [52].

Better results were obtained when purified population of MSCs was used for grafting. Such a survey was performed by Horwitz *et al.* in 2002 [50], demonstrating the successful engraftment of MSCs. The study enrolled six children, each of them received two infusions of the allogenic cells. MSCs were transduced with the LNc8 or G1PLII retroviral vectors, in order to localize the engrafted cells in patients. The vectors contained either the neomycine phosphotransferase gene (neo^R) or nonexpressing β-galactosidase (β-gal) and neo^R sequences, respectively. The transduction efficiency was in a range from 2% to 25%. The cells expressing G1PLII marker were detected in five patients, at least at one site. The localization included bones, skin and marrow stroma and brought a positive healing effect expressed as the acceleration of growth velocity, in a range from 60% to 94% of the predicted values for age- and sex-matched healthy children [50].

Furthermore, there has been a novel clinical trail of *in utero* MSC transplantation in patient with severe OI [73]. Allogenic, HLA-incompatible MSCs obtained from a human male fetal liver, were injected to the umbilical vein at the week 32 of gestation, in a total number of 6.5×10^6 cells. After a baby-girl delivery, a centromeric XY-chromosome-specific probe revealed 0.3% of the donor cells. Interestingly, when examining whole male genome, the detection of Y chromosome-positive cells showed 7.4% of the engraftment. There was no immunoreactivity against transplanted cell detected, indicating the safety of the study. The outcome was outstanding, demonstrating the improvement of bone mineralization from 48% at 3 months to 56% at 12 months and 76% at 22 months, in comparison to age-matched controls. However, this increase may be partially attributed to pamidronate treatment, started from the 4th month. The follow-up revealed only three fractures during the first two years, normal psychomotor development and correct growth tendency.

A new approach toward OI treatment has been developed with the occurrence of gene therapy. In the picture of the disease, the product of mutant allele interferes with the peptide produced by normal allele, resulting in abnormal collagen fibril formation. The gene therapy therefore, should be first directed toward silencing of the mutant allele expression, and then replacing the mutated gene. This can be achieved either by degradation of the mutant mRNA or by disruption of the mutant gene [12]. However, the treatment strategy might be complicated by the genetic heterogeneity of the disease and the fact, that most OI mutations are dominant-negative.

Gene therapy trial combined with the use of MSCs was performed by the Russel's group [12], who performed *ex vivo* genetic modification of autological MSCs from OI patients. The cells were targeted with viral vector AAV-COLe1INpA that was designed to disrupt exon 1 of the chromosomal *COL1A1* gene, by inserting an inactivating cassette. This would change the mutated gene into a null form, eliminating the production of abnormal collagen chains, thus leading to mild disease symptoms. The results demonstrated that 31% to 90% of the positively selected MSC clones (0.06% to 0.23% of unselected MSCs) underwent gene targeting at one allele of *COL1A1* gene. There were very similar targeting frequencies at mutant and wild-type alleles, suggesting that there was no allele preference in this process. Furthermore, very similar targeting frequencies in a range of 90% were observed in polyclonal, as well as in monoclonal cell population. Gene modification improved collagen processing, stability and structure, thus preventing pro-collagen peptide retention within the cells. Moreover, the diameter of collagen fibrils, as well as the melting temperature was dramatically improved, resembling the values obtained for wild-type cells. The targeted cells were also tested for bone and fat formation ability *in vivo*, demonstrating their multilineage potential.

Another great challenge for tissue engineering using MSCs is the treatment of cartilage lesions. The first reports handling this issue come from Wakitani *et al.* [124], who filled mechanically induced full-thickness lesions in New Zealand white rabbits with collagen sponges saturated with MSCs. These cells differentiated into active chondrocytes that produced cartilagineous matrix. However, there were some drawbacks in the first experiment: a discontinuity between the host tissue and the new tissue, as well as the progressive thinning of the repaired tissue was observed [14]. Other scientists successfully performed the cartilage differentiation in knee joints, using MSCs stimulated with BMP-2 and IGF-1 [40], whereas unstimulated MSCs failed to induce chondrogenesis under the same circumstances [89]. It is also worth to itemize that pro-inflammatory cytokines, which are expressed in abundance in pathological situations, effectively inhibit BMP-mediated chondrocyte response. Nevertheless, there have been reports of MSC differentiation

into tendon [131], as well as trials for vertebral disc regeneration with the use of scaffolds [104]. Those animal model results seem to be very promising, however, further studies are needed before their application to humans.

Further example of potential clinical MSC usefulness is the possibility to accelerate the reconstitution of hematopoiesis in patients after myeloablative chemotherapy or radiotherapy. Such approach seems to successfully attenuate graft versus host disease (GvHD) after hematopoietic stem cell transplantation. The stromal support has been well documented to be essential for hematopoiesis and the cell-cell interactions in the marrow microenvironment are critical for normal hematopoietic function [123]. In a mouse model, MSC infusion not only prevented the occurrence of graft failure, but also had an immunomodulatory effect [39]. Moreover, preliminary reports of co-transplantation of MSCs and HSCs from HLA-identical siblings showed the reduction in acute and chronic GvHD [72]. It was demonstrated that addition of MSCs to the grafting material significantly accelerated reconstitution of hematopoiesis in autologic and allogenic transplantations. This was observed especially in umbilical cord blood transplantation, both haploidentical and from unrelated donors [36]. In one case report, a patient with acute lymphoblastic leukemia, who developed severe GvHD after allogenic HSC transplantation and did not respond to the applied therapy, was cured by the use of haploidentical MSCs. The cells were given twice and no toxicity after infusion was observed. The outcome indicated that MSCs had a striking immunosuppressive effect and caused a rapid healing of damaged gut epithelium. Additionally, the patient had no minimal residual disease in blood and bone marrow one year after transplantation [74].

In addition, there are also observations indicating the usefulness of MSC transplantation in myocardium regeneration after myocardial infarction. Among all bone marrow-derived cell populations, only MSCs were shown to be able to differentiate into cardiomyocytes *in vitro* [45]. Murine model studies using 5-azacytidine to induce cardiomyocyte differentiation confirmed at the molecular level that this cell type could originate from MSCs [80]. The cells not only contained myotube-like structures and myofilaments, but were also positively stained for the cardiomyocyte-specific markers, such as sarcomeric myosin, desmin and actinin, and showed the expression of cardiomyocyte-specific genes and transcription factors [80]. The same effect was obtained for human MSCs [128]. Prompted by *in vitro* studies, scientists performed *in vivo* experiments. Wang *et al.* [126] demonstrated that murine MSCs participate in the formation of new cardiomyocytes in the normal, uninjured heart. Immunohistochemistry executed 4 weeks after injection proved that donor-derived MSCs were present in the heart, expressing cardiac markers. The same potential was demonstrated for human MSCs, which were injected into the heart of SCID mice. Although the cells engrafted in small percentage (0.44%), they were positive for cardiac markers [118]. When used in animal models for cardiac damage, MSCs successfully colonized the injured tissue and transformed into properly active cardiac cells [119]. Spectacular results were obtained when MSCs transplanted into injured heart were transduced with a virus encoding Akt - an anti-apoptotic gene prolonging cell survival, which prevented the pathological remodeling of the left ventricle after infarction. Approximately 80% of the injured myocardium regenerated and the cardiac function was completely restored [81]. Besides improving cardiac function, MSCs were shown to be able to increase the ventricular wall mass [113]. Furthermore, local administration of MSCs to the heart generated *de novo* myocardial formation, giving the hope of the use of these cells in the treatment of coronary heart disease [90]. The injection of MSCs into infarct zone of patients with myocardial infarction appeared to be beneficial for the general heart functionality [115].

Promising results have been also obtained when using MSCs in neuronal lesion treatment. Previous studies showed that MSC transplantation improves recovery after stroke or traumatic brain injury [16]. Additionally, in *in vitro* co-cultures of MSCs and neural stem cells, preferential neuronal differentiation has been observed [77]. Moreover, grafts of MSCs in animal models have been shown to promote remyelination [1] as well as partial recovery of function [17]. After direct injection of MSCs into rodent brain, the cells migrated within the brain and differentiated into $GFAP^+$ glial populations [4]. The transplantation of MSCs into infarcted brain led to the reduction of cell death and the increase in cell proliferation. Moreover, MSCs were demonstrated to be able to produce even myelinating Schwann-like cells, with the typical spindle-shaped morphology and the expression of specific markers, such as LNGFR, Krox-20, CD104 and S100 [65]. Testing these cells *in vivo*, by means of transplantation to autologous muscle conduit with 2 cm gap in rat sciatic nerve, showed their capacity to colonize the lesion site and regenerate the damaged nerve. The cells were able to myelinate more than one axon in some cases, similarly as it is in CNS [65]. In a different set of experiments, MSCs transplanted into a subtotal cervical hemisection in adult female rats, were able to integrate efficiently into the injury site. Moreover, immunohistochemical analysis showed marked axonal growth, indicating that these cells enhance axonal growth after spinal cord injury. Interestingly, the recovery levels strongly depended on the human donor and even varied from lot to lot of MSCs isolated fraction [87].

The list of reports indicating that MSCs contribute to tissue repair *in vivo* enlarges. There are examples of MSC utilization in the repair of kidney [47], muscle [23] and lung [91]. The cells were also found to promote angiogenesis [46], and were used in chronic skin wound treatment [6]. The implantation of MSCs together with occlusive dressing and subsequent epidermal grafts significantly accelerated wound healing and decreased the risk of amputation in endangered patients [130].

Clinical trials based on MSCs can omit many of the limitations associated with the use of embryonic stem cells (ES). Unlike ES, MSC are not immunogenic, when used autologically, they do not induce immune rejection and are also less probable to trigger teratoma formation, not to mention the ethical concerns.

Unfortunately, there are also some drawbacks concerning the use of MSCs. Firstly, according to some observations MSCs fused with endogenous differentiated cells and formed tetraploid cells *in vivo*, although such an event seems to be extremely rare [114]. Secondly, MSCs were shown to permit tumor growth in allogenic recipients [30] in animal models. A further question arises, whether the grafted MSCs can maintain their undifferentiated state, thus supporting the therapeutic effect on a long term basis.

Concluding remarks

It seems well-founded that MSCs constitute a superb potential tool in regenerative medicine and gene therapy approaches. They possess an extensive proliferative potential and are able to differentiate into various cell lineages. Due to these important features, the use of MSCs in clinical trials increases. It has been documented that these cells engraft successfully in patients and cause beneficial effects. After learning more about their properties, it will be possible to start new, more advanced and better treatment strategies for various diseases, even those, which seem to be incurable at present. Moreover, knowing that each patient is genetically different and may give different response to a treatment, and carry variable predisposition to different diseases, specifically targeted strategies using autologous MSCs, may be designed. However, it is still a long way to go before using these cells as a routinely applied therapy in clinics.

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